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Prophage Substitution and Prophage Loss from Superinfected *Escherichia coli recA*(P1) Lysogens

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It is shown that the plasmid prophage P1 can be displaced by a superinfecting P1 phage in *Escherichia coli recA*(P1) lysogens. Six widely separated phage markers were used to distinguish between residual recombination and total substitution. It is further shown that superinfection of *recA* lysogens can lead to loss of both phage (curing). These two phenomena, previously reported in Rec$^+$ strains, are thus independent of host recombination and may result from perturbations of some function involved in plasmid maintenance.

Two P1 plasmid phage cannot be stably maintained together in the same cell in the absence of any selective pressure (a manifestation of plasmid incompatibility). Studies in Rec$^+$ lysogens (9) have shown that when a superinfecting phage marker is selected, concomitant loss of a resident prophage marker is observed. This could result from reciprocal recombination between the superinfecting and resident genomes or from prophage substitution.

This report establishes that P1 prophage substitution occurs efficiently in *recA*(P1) lysogens. The bacterial mutations *recA* or *recB* lower recombinant formation during bacteriophage P1 crosses (4); lysogenization in strains bearing these mutations is still possible, although less efficient (10). To distinguish between residual recombination and prophage substitution, multiply marked phage were used (see Fig. 1). Lysogens were superinfected, and clones selected for one marker of the superinfecting phage were tested for the other markers.

In the course of this work, it was found that prophage loss (curing) can occur as a result of superinfection of *recA*(P1) lysogens, as has been shown to occur in superinfected Rec$^+$(P1) lysogens (9).

MATERIALS AND METHODS

Bacteria and phage. All bacterial strains used were derivatives of *Escherichia coli* K-12. Lysogenization and superinfection experiments were carried out in the *supE recA13* strain AB2463 (6). Since *recA* strains contain nonviable bacteria, the otherwise isogenic *recA*$^+$ strain AB1157 (5) was also lysogenized and used to calculate the total number of cells for a given optical density. C600 (1) was used for growth and assay of phage. The *sup*$^+$ (nonsuppressing) *recA* strain N100 (16) served to control the lysogenization ability of the phages used. The *sup*$^+$ strain Q125 (2) was used to score the amber markers.

The phage P1Cm *am62 am3.6 am8.13 c1.100* and P1Km *am32 c1.100* were constructed in standard crosses from P1Cm *c1.100* (10), P1Km (12), and P1 amber mutants *am62*, *am3.6*, *am32* (15), and *am8.13* (11). Where necessary (scoring of the amber markers, immunity tests), the single amber mutants were used along with P1*vir am30* (from D. H. Walker, Jr.) and P1*c1.100*. All amber mutations used are suppressed by *supE*. Cm and Km are chloramphenicol and kanamycin resistance genes acquired from R factors by illegitimate recombination (8, 12); these insertions do not interfere with phage lysogenization or vegetative growth. They serve as convenient selective indicators of prophage. P1Cm *c1.100* carries a temperature sensitive mutation in the *c1* repressor gene; lysogens for this phage are induced above 35°C (10).

Media. LB broth for liquid culture, solidified LB for plating bacteria, LMC plates for P1, and P1 dilution medium of Walker and Anderson (14) for dilutions are described in D'Ari et al. (2). Where necessary, media were supplemented with thymine (25 μg/ml), citrate (5×10^{-3} M), and the antibiotics chloramphenicol (Roussel UCLAF, S.A.) and kanamycin (Sigma Chemical Co.) at a final concentration of 25 μg/ml each.

Assay of bacteria: growth and assay of phage. Bacteria were titrated as colony formers in agar overlaid on solidified LB. The presence of the *recA* mutation was verified by sensitivity to UV radiation.

Phage stocks were prepared from isolated single plaques by confluent lysis (2). Since the Cm and Km insertions are unstable during vegetative growth, our stocks were tested; those used here were 92% Cm^r and 97% Km^r. They were sterilized with chloroform and contained no viable bacteria. Phage assays were carried out by preadsorption of 0.1 ml of an appropriate

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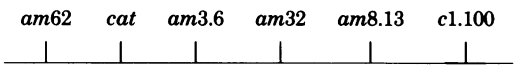

FIG. 1. *Genetic map of P1Cm (not drawn to scale), showing the relative order of the mutations used in this work (2). The cat gene confers chloramphenicol resistance; the Km insertion has not been mapped.*

dilution of P1 to 0.1 ml of a late-exponential C600 culture in 2.5×10^{-3} M $CaCl_2$ for 10 min at 37°C, followed by plating with 3 ml of top agar on LMC plates and overnight incubation at 37 or 42°C.

Lysogenization: superinfection of lysogens. Overnight LB cultures of the desired strains were diluted 30-fold into fresh medium at 30°C, grown to an absorbance at 650 nm of 0.3, concentrated threefold by centrifugation, and suspended in LB with 2.5×10^{-3} M $CaCl_2$ at 2×10^7 to 5×10^7 viable cells per ml. A 0.1-ml amount of an appropriately diluted P1 suspension was added to 0.9 ml of cell suspension. After 30 min of adsorption at 30°C with gentle agitation, unadsorbed phage were removed by centrifugation, cells were resuspended in LB containing 5×10^{-3} M citrate, and portions were plated on LB plus citrate with or without antibiotics. The citrate prevented readsorption of phage liberated during incubation.

Scoring of the markers. Lysogenic clones to be tested were picked into 0.4 ml of LB in nylon trays (2) and incubated for 7 h at 30°C. To test the drug resistance markers, the cultures were replicated, 25 at a time, onto plates containing kanamycin, chloramphenicol, both drugs, or no drug. The cultures were then diluted about 100-fold into 0.4 ml of LB containing 2.5×10^{-3} M $CaCl_2$ inoculated with 10^6 C600 cells and incubated at 42°C for 2 h; this induced the lysogens and permitted further multiplication of the phage released. Amber markers were scored by replication from these trays (or from 100-fold dilutions) onto LMC plates seeded with 0.1 ml of a late-exponential culture of the *sup*$^+$ strain Q125 and 3×10^7 P1 amber tester phage; the plates were then incubated overnight at 42°C. Lysis indicated the presence of the wild-type allele of the amber mutation carried by the tester phage.

Characterization of nonlysogenic bacteria. All prophage used bear the *c*1.100 mutation and one antibiotic resistance marker, Cm or Km. Spontaneous nonlysogenic segregants were selected by their ability to form colonies at 42°C on LB citrate plates; they were tested for their antibiotic sensitivity and UV sensitivity (to confirm the presence of the *recA* mutation). Nonlysogens arising after superinfection were detected as clones sensitive to the antibiotics used; they were tested for UV sensitivity, for thermoresistance in LB at 42°C, and for loss of immunity. Although P1 cannot form plaques on *recA* lawns (3), the following immunity test distinguishes between lysogens and nonlysogens: 0.1 ml of a late-exponential culture was spread with top agar on LMC plates and incubated at 32°C with spots of P1Cm *c*1.100 at concentrations ranging from 10^7 to 10^9 per ml. Circles of lysis indicated nonimmunity. A P1 lysogen of AB2463 *recA* was used as immune control.

RESULTS AND DISCUSSION

The *supE recA* lysogen AB2463(P1Km *am*32 *c*1.100) was superinfected by phage P1Cm *am*62 *am*3.6 *am*8.13 *c*1.100 (Table 1, experiments 1, 2, and 3). The Cm marker of the superinfecting phage was used to select 25 Cm^r colonies in each experiment. These clones were grown and analyzed for the five unselected markers. They proved to have acquired all the markers of the superinfecting phage—Cm (by selection) and all unselected markers (the three amber markers and the allele *am*32$^+$)—and to have lost the Km marker (except for experiment 3, in which one clone was Km^r) and the dominant alleles of the original prophage (our tests do not permit us to detect recessive markers when the dominant alleles are present).

Similarly, in the reciprocal experiments [Table 1, experiments 4, 5, and 6; *supE recA* strain AB2463(P1Cm *am*62 *am*3.6 *am*8.13 *c*1.100) superinfected by P1Km *am*32 *c*1.100], the 25 Km^r clones tested in each experiment had acquired all five unselected markers of the superinfecting phage and lost the Cm^r marker and the dominant *am*32$^+$ allele of the prophage.

Since P1 prophage can be lost spontaneously (10), lysogenic cultures include nonlysogenic cells. The possibility thus arises that the bacteria carrying all the markers of the superinfecting phage and no detectable marker of the original prophage could be the result of lysogenization of nonlysogens and not due to displacement of the established plasmid by the superinfecting phage. The following observations show that this was not the case. The strains AB2463(P1Km *am*32

TABLE 1. *Prophage substitution and recombination frequencies after superinfection*[a]

Superinfecting phage	Prophage	Expt no.	Multiplicity[b]	Frequency of lysogens selected for the superinfecting resistance marker[c]
P1Cm *am*62 *am*3.6 *am*8.13 *c*1.100	P1Km *am*32 *c*1.100	1	20	1.5×10^{-2}
		2	1.3	0.13×10^{-2}
		3	0.13	0.11×10^{-2}
P1Km *am*32 *c*1.100	P1Cm *am*62 *am*3.6 *am*8.13 *c*1.100	4	13	20×10^{-2}
		5	2.5	9×10^{-2}
		6	0.25	0.7×10^{-2}

[a] The superinfection experiments were carried out as described in the text. In experiments 3 and 6, cultures were pregrown in LB plus kanamycin or chloramphenicol, respectively.

[b] Each value indicates the number of adsorbed phage per cell. Unadsorbed phage were assayed at 30°C to avoid induction of any remaining lysogens in the supernatant; correction was made for the efficiency of plating at 30°C, 0.6 for P1Km *am*32 *c*1.100 and 0.25 for P1Cm *am*62 *am*3.6 *am*8.13 *c*1.100.

[c] Frequencies are normalized to the number of infected bacteria, calculated by the Poisson distribution.

*c*1.100) and AB2463(P1Cm *am*62 *am*3.6 *am*8.13 *c*1.100) segregate nonlysogenic cells at frequencies of 4×10^{-4} and 2×10^{-5}, respectively. Lysogenization experiments, carried out under the same conditions as the superinfection experiments, showed that P1Cm *am*62 *am*3.6 *am*8.13 *c*1.100 and P1Km *am*32 *c*1.100 lysogenize AB2463 at frequencies of 1×10^{-3} and 9×10^{-2}, respectively. Thus, the nonlysogenic fraction of the population lysogenized by the infecting phage in each case is many orders of magnitude below the frequency of Cm^r or Km^r colonies recovered.

Our stocks were grown on Rec^+ strains. P1, a generalized transducing phage, introduces the *recA*$^+$ gene at a frequency of 2×10^{-4} per infecting phage (3). This again is much lower than the frequency of Cm^r or Km^r colonies found after superinfection. Thus, the substitution observed—acquisition of all markers of the superinfecting phage and loss of markers of the initial prophage—is not dependent on a *recA*$^+$ transducing phage coinfecting with another P1 phage.

Several differences were observed between the two phage used. The two lysogens differed in their ability to produce infective centers: the ratio of plaques on C600 at 42°C to viable bacteria at 30°C was 0.3 for AB2463(P1Cm *am*62 *am*3.6 *am*8.13 *c*1.100) and 0.9 for AB2463(P1Km *am*32 *c*1.100). Furthermore, the P1Cm derivative substitutes for a resident prophage less efficiently than the P1Km derivative and shows lower frequencies of lysogenization. This is unlikely to be due to poor suppression of one of the amber mutations, since both of these phage can lysogenize the *sup*$^+$ *recA* strain N100.

In the superinfection experiments, the bacteria analyzed were selected on chloramphenicol or kanamycin plates. To show that the substitution observed was not induced by the selection employed, colonies arising on plates containing no antibiotic were also analyzed for the presence of the Cm and Km markers. The frequency of Cm^r colonies (4% for experiment 1) or Km^r colonies (18% for experiment 4 and 7% for experiment 5) was in agreement with the frequencies of Cm^r and Km^r clones selected directly after superinfection.

Unexpectedly, Cm^s Km^s clones were also detected among the colonies tested in experiments 4, 5, and 6, at frequencies similar to the substitution frequencies (2/33, 3/43, and 1/100, respectively) and at least 500-fold higher than the spontaneous curing frequency. These Cm^s Km^s clones were shown to be *recA* nonlysogens (UV sensitive, nonimmune, temperature resistant). These results show that a *recA*(P1) lysogen can be cured of its prophage by superinfection with another P1. The phenomenon of prophage curing in *recA* lysogens has subsequently been confirmed by A. Jaffé-Brachet and S. Briaux, who have extensively analyzed the kinetics of appearance of cured cells, detected as temperature-resistant colony formers (manuscript in preparation).

Mise and Arber (9) studied the possibility of maintaining two P1 prophages together in the same cell and reported that rare unstable double lysogens could be selected in Rec^+ bacteria (frequencies around 10^{-3}) but that when selection was removed, these segregated single lysogens and nonlysogens. The results presented here show that the phenomena of prophage substitution and curing do not depend on the *recA*$^+$ function. As for the formation of unstable double lysogens, the frequency of Km^r Cm^r clones in superinfected *recA* lysogens is less than 10^{-5} (data not shown), suggesting that the double lysogens observed by Mise and Arber were formed by homologous recombination (insertion of one P1 genome into the other) and that little recombination takes place under our conditions, although we cannot eliminate the possibility that double-length plasmids are formed by an illegitimate reciprocal recombination event but are not maintained, even unstably, in *recA* cells. In similar superinfection experiments with Rec^+ bacteria we obtained 20% Km^r Cm^r stable recombinants (both resistance markers on a single phage genome), which masked any rare unstable double lysogens; it is possible that the new Cm and Tc insertions used by Mise and Arber are tightly linked or allelic, thus preventing normal recombinant formation in their experiments.

Superinfection of a P1 lysogen apparently destabilizes the resident prophage; it can lead to prophage substitution or loss of both phage. Destabilization of a resident plasmid in the presence of an abnormally high number of plasmid copies per bacterial chromosome has been reported for mutants of R1 (13). It is possible that a similar mechanism operates in these different situations (7). Our experiments were carried out in the absence of recombination and under conditions of prophage immunity. This suggests that prophage displacement and curing may depend on some function expressed in lysogens, perhaps a plasmid maintenance function.

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